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Asymmetric Expression of the *SpHE* Gene along the Sea Urchin Embryo Animal–Vegetal Axis

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The mechanism that establishes the maternally determined animal–vegetal axis of sea urchin embryos is unknown. We have analyzed the *cis*-regulatory elements of the *SpHE* gene of *Strongylocentrotus purpuratus*, which is asymmetrically expressed along this axis, in an effort to identify components of maternal positional information. Previously, we defined a regulatory region that is sufficient to provide correct nonvegetal expression of a β-galactosidase reporter gene (Wei, Z., Angerer, L. M., Gagnon, M. L., and Angerer, R. C., *Dev. Biol.* 171, 195–211, 1995). We have now analyzed this region intensively in order to determine if the spatial pattern is controlled by nonvegetal-positive activities or by vegetal-negative activities. The regulatory sequences, except the basal promoter, were mutated by either deletion or sequence replacement. None of these mutations resulted in ectopic β-gal expression in vegetal cells, showing that no single negative *cis* element is responsible for the lack of vegetal *SpHE* transcription. Surprisingly, even short segments of the regulatory region containing only several identified *cis* elements also direct nonvegetal expression. Furthermore, the *SpHE* basal promoter functions effectively in vegetal cells in combination with *cis*-acting elements derived from the PMC-specific gene, *SM50*. We conclude that the spatial pattern of *SpHE* transcription is achieved by multiple positive activities concentrated in nonvegetal cells. The vegetal expression of *SM50* also is regulated only by positive activities (Makabe, K. W., Kirchhamer, C. V., Britten, R. J., and Davidson, E. H., *Development* 121, 1957–1970, 1995). A chimeric promoter containing both *SpHE* and *SM50* regulatory sequences is active ubiquitously, suggesting that these regulators are not reciprocally repressive. These observations suggest a model in which the *SpHE* and *SM50* genes are activated by separate sets of positive maternal activities concentrated, respectively, in nonvegetal and vegetal domains of the early embryo.

INTRODUCTION

The *SpHE* gene of the sea urchin *Strongylocentrotus purpuratus* is one of a set of genes expressed zygotically only at the very early blastula (VEB) stage (Reynolds *et al.*, 1992). Most interestingly, like other genes of this group, the early, transient expression of *SpHE* reflects the maternally specified animal–vegetal axis because transcripts accumulate in most blastomeres, but are excluded from about 10 to 15% at the vegetal pole. This nonvegetal expression pattern is very likely regulated by maternally positioned transcriptional regulatory activities asymmetrically distributed along the animal–vegetal (A–V) axis because VEB mRNA accumulation is autonomous in cells separated continuously beginning at the two-cell stage (Reynolds *et al.*, 1992; Ghiglione *et al.*, 1993). Furthermore, the expression of a *SpHE* homolog in another sea urchin species is not modulated by inductive influences from transplanted micromeres, consistent with the proposition that the animal–vegetal regulation of these genes is mediated by maternal factors that act independently of cell–cell interactions (Ghiglione *et al.*, 1996). Therefore, *SpHE* and other VEB genes provide useful tools for studying the mechanism that establishes the A–V axis of the sea urchin embryo. We are analyzing the *SpHE* transcription apparatus in an attempt to understand the molecular basis of this asymmetry.

Previously, we demonstrated that *SpHE* regulatory sequences within about 300 nt upstream of the transcription start site are necessary and sufficient for regulating nonvegetal expression of a β-galactosidase (β-gal) reporter gene. In this defined regulatory region, multiple *cis* elements were identified biochemically and functionally, including SpOtx, ets, and CCAAT sites as well as several unknown sequences. Mutational analysis showed that the *cis* elements providing high-level expression were redundant for quanti-

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tative output of the transgenes because several different subsets could drive transcription at similar, very high levels.

Two major mechanisms might confer the *SpHE* nonvegetal expression pattern. Either positive transcription activities are absent from, or inactive in, the vegetal pole or negative activities are concentrated there. Here we report our study of these two possibilities and find that the nonvegetal *SpHE* expression pattern is most likely regulated by multiple nonvegetal localized positive transcription activities.

MATERIALS AND METHODS

Preparation of SpHE Promoter/β-Galactosidase Transgenes

All transgenes used in this work are diagrammed in Fig. 1. All *SpHE* promoter sequences were inserted into the β-gal expression vector, PNL (Gan *et al.*, 1990), between *Sph*I and *Sal*I restriction sites. The inserts were DNA fragments produced by restriction enzyme digestion or PCR. For *SM50*–PNL, the promoter sequence from −440 to +120 was inserted between the *Hin*dIII and *Sal*I sites. For the *SpHE–SM50* chimera, SS-91, *SM50* sequence from −10 to +29 was substituted with *SpHE* sequence from −91 to +96; for SS-1255, the *SpHE* fragment of −1255 to −33 was placed just 5′ of *SM50*–PNL. The replacement mutation constructs were made as described previously (Wei *et al.*, 1995). Sequence of SH + 20R, SHΔ-240-92R, and SH-147R from −72 to −40 was replaced with the same length sequence, TCAGTCATGTACCAGAATTCTGGTACATGACTA. Each construct was confirmed by sequencing.

Assay of β-Galactosidase Activity in Sea Urchin Embryos

Transgenes were introduced into sea urchin eggs by microinjection and transgene expression was analyzed as previously described (Wei *et al.*, 1995). All transgenes, except SH-147R, were tested in multiple egg batches as indicated in Fig. 1. Briefly, about 2500 copies of linearized plasmid DNA were injected into *S. purpuratus* eggs just after fertilization that were then cultured in artificial seawater at 15°C for about 40 hr to midgastrula stage. To detect β-gal expression, embryos were fixed in 2% paraformaldehyde (in seawater) for 6 min, washed with staining buffer, and stained with 0.15% X-gal. The staining time was varied from 4 to 48 hr, depending on the strength of promoters. In order to recognize vegetal pole-derived cells, a PMC-specific antibody, 6e10, kindly provided by Dr. C. Ettensohn, Carnegie–Mellon University, was used as previously described (Wei *et al.*, 1995). Briefly, X-gal-stained embryos were washed with seawater and labeled with Cy3-conjugated antibody in the presence of 0.25% Triton X-100 overnight at room temperature. The X-gal and 6e10 doubly stained embryos were examined with a Nikon inverted microscope using DIC and fluorescence optics. Examples illustrated were photographed using Ektachrome 1600 (Kodak, Rochester, NY) slide film. Films were scanned with an AFGA Arcus II scanner at 1200 dpi and processed using Adobe Photoshop 3.0.5.

RESULTS

No Single cis Element Outside the Basal Promoter Is Required to Suppress SpHE Expression in PMCs Derived from the Vegetal Pole

As mentioned above, the nonvegetal expression pattern of the *SpHE* gene might be conferred by one or more negative factors functioning in cells of the vegetal pole region. In our previous analysis, when some of the identified *cis* elements were mutated, no detectable increase of promoter activity was observed using a CAT reporter gene assay (Wei *et al.*, 1995). However, since the *SpHE* gene is inactive in only 10–15% of the embryo, any increase in CAT activity in that region resulting from mutation of a negative element would be within the error of the assay and therefore not detectable. We therefore analyzed *SpHE* promoter activity at the single-cell level by using β-gal as the reporter. If a negative element were deleted from, or replaced in, the *SpHE* regulatory region, and if that were the only functional negative element, then we would expect to see ectopic expression of β-gal in primary mesenchyme cells (PMCs) derived from the vegetal pole as well as normal expression in ectoderm and endoderm cells. We carried out all of these assays at midgastrula stage for several reasons. First, the promoter activity of the control PMC-specific construct, *SM50*-PNL, is higher at gastrula stage than at blastula stage (Makabe *et al.*, 1995). β-Galactosidase signals for *SpHE* constructs are similar at these two stages, partly because microinjected *SpHE* promoter constructs remain active after the endogenous genes have shut down (Wei *et al.*, 1995) and partly because β-galactosidase is stable in sea urchin embryos (Gan *et al.*, 1990). Second, we wanted to maximize the sensitivity of detection of ectopic *SpHE* promoter activity in PMCs by allowing more time for β-gal accumulation. As discussed in greater detail previously (Wei *et al.*, 1995), embryos containing β-gal-positive cells in ectoderm and/or endoderm are scored as having nonvegetal expression (Fig. 1, NV); those containing labeled PMCs are considered to have vegetal expression (Fig. 1, V). PMCs were distinguished from abnormal internal cells, which are frequently observed in injected embryos, by staining with the PMC-specific antibody, 6e10 (Wei *et al.*, 1995). The domains of nonvegetal and vegetal expression are illustrated in Fig. 2A.

Initially we tested all sequences along the *SpHE* regulatory region from −310 to +96, except those containing the basal promoter, by 5′, 3′, or small internal deletions. Figure 1A shows the structure of each construct and the results of these assays. In SH+20R, sequence from −72 to −40 was mutated by replacement, rather than by deletion, in order to maintain correct spacing between upstream elements and the basal promoter. If the promoter is active in all cell types, then β-gal expression in PMCs would be expected to occur, on average, in about 25% of embryos (Makabe *et al.*, 1995) while that in ectoderm/endoderm cells would be considerably higher (>75%). This is because incorporation of exogenous DNA in blastomeres of different embryos is

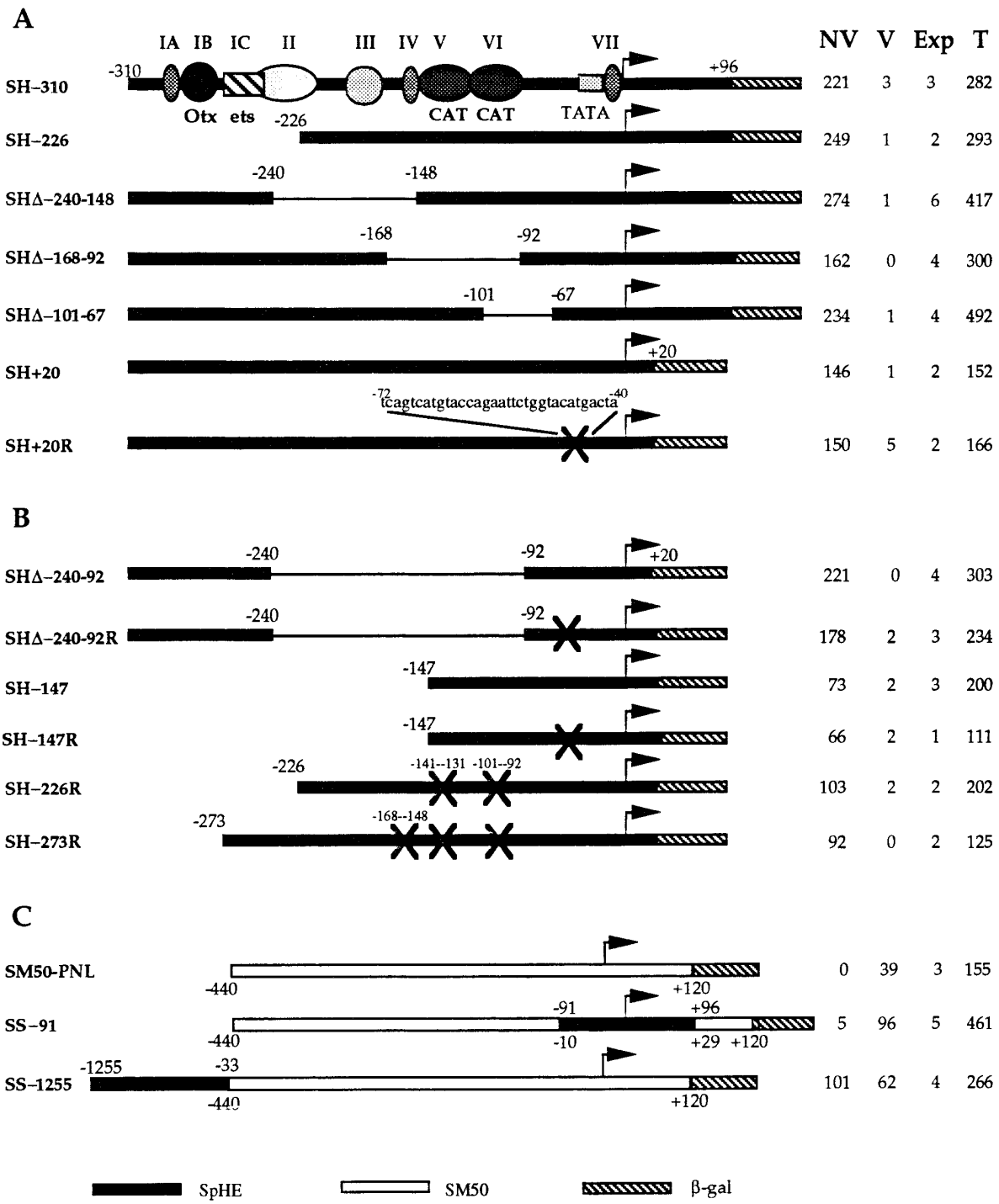
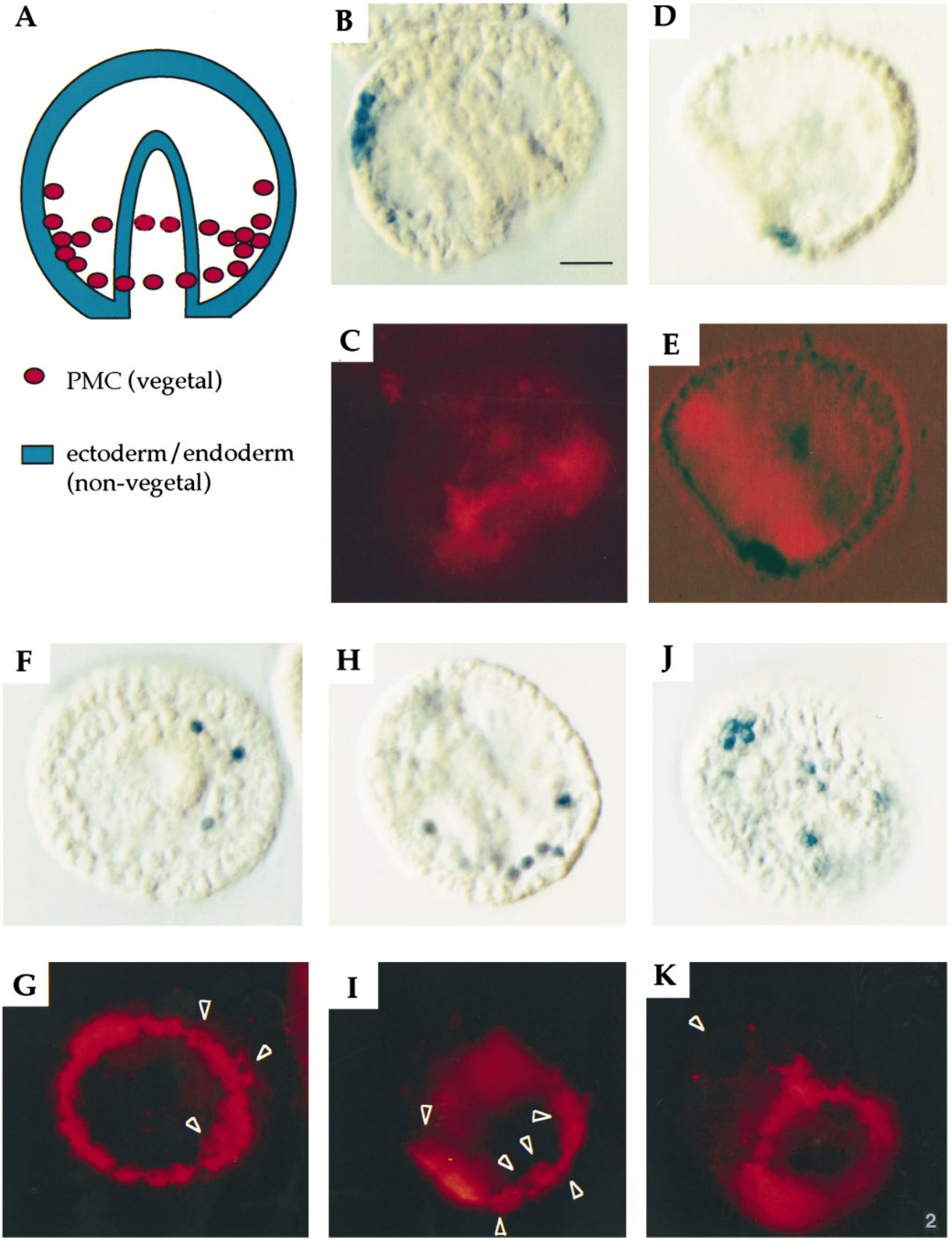


FIG. 1. Schematic diagrams of injected constructs and tabulation of β-galactosidase expression patterns revealed after X-gal staining. *cis* elements identified previously (Wei *et al.*, 1995) are indicated in the wild-type construct SH−310 by Roman numerals. Elements that bind the same factors are indicated with the same symbols and several identified factors are named (Otx, ets, CCAAT). Thick bars represent DNA sequences, filled for *SpHE*, open for *SM50*, and hatched for β-gal. Thin lines represent deleted sequences. Arrows indicate the transcription start sites and X represents sequence replacement sites. The numbers above and below the bars indicate the nucleotide positions of *SpHE* and *SM50* sequence relative to the transcription start site, respectively. T is the total number of injected eggs and Exp is the number of different egg batches used. V is the number of embryos that contained β-gal-positive PMC cells, which represents the vegetal pattern. The identity of PMCs was confirmed by staining with antibody 6e10. NV is the number of embryos that had β-gal-positive cells in ectoderm and/or endoderm, which represents the nonvegetal pattern. (A) *SpHE* promoter constructs with short sequences deleted or replaced. The replacement in SH + 20R is between −72 and −40. (B) *SpHE* promoter constructs containing large deletions and site replacements. The replacement in SHΔ-240-92R and SH-149R is the same as that of SH+20R. The replacements in SH−226R are at the CCAAT boxes, sites V and VI, and those in SH-273R are at IV, V, and VI. (C) *SM50* and *SpHE*/*SM50* chimeric constructs.


A
PMC (vegetal)
ectoderm / endoderm
(non-vegetal)
B
C
D
E
F
H
J
G
I
K
2

randomly mosaic and occurs most frequently between the 8- and 32-cell stages (Hough-Evans *et al.*, 1988; Livant *et al.*, 1991). For all six constructs shown in Fig. 1A, we saw a very high frequency of embryos containing labeled nonvegetal cells, as previously reported for the intact promoter (Wei *et al.*, 1995). In several cases, the frequency of labeling was reduced slightly (i.e., to between ~45 and 65%), undoubtedly because the output of β-gal from weaker promoter constructs in some cells is below the detection limits of the assay.

Only a very low percentage of embryos expressing these constructs, around 1–3% of injected embryos, had β-gal-positive PMCs. It is unlikely that this low level of ectopic expression is caused by deletion or mutation of a specific sequence because it is observed with almost all constructs. Instead, we believe that the low level of *SpHE* promoter activity in PMCs represents the noise level of this assay. First, the frequency of expression in PMCs is an order of magnitude lower than ~25% observed with the PMC-specific *SM50*-PNL control construct (Fig. 1C; Makabe *et al.*, 1995). Second, the frequency of PMC labeling varies among batches of sea urchin eggs, and no PMC labeling is observed in some experiments. We note that the frequency of labeled PMCs is even lower when the assay is carried out 28 hr after fertilization (Wei *et al.*, 1995; Wei, unpublished observations), consistent with the interpretation that the activity seen at 40 hr results from progressive accumulation of extremely low levels of β-gal in a few embryos. We conclude that the activity of each of these partial *SpHE* promoters is nonvegetal. This result suggests that no single negative element in *SpHE* regulatory sequence is sufficient to mediate nonvegetal transcription of this gene.

Small Subregions of the SpHE Promoter Drive Low-Level Expression Which Remains Nonvegetal

In order to investigate the possibility that *SpHE* spatial regulation is conferred by multiple negative elements, each of which is sufficient to repress transcription in vegetal cells, we further reduced the amount of potential regulatory sequence by additional deletions or *cis*-element replacements (Fig. 1B). Each of these constructs contains only a small segment of the regulatory region plus the basal promoter sequence, which is defined here as TATA and the initiator region to +20. As expected from previous analyses with the CAT reporter (Wei *et al.*, 1995), the activities of these reduced promoters were lower: Fewer cells were β-gal positive, a lower percentage of embryos was labeled, and longer times for X-gal staining were required. Nevertheless, signals were sufficient to determine that the expression of each of these constructs was also nonvegetal; again only very low levels of ectopic expression (<3%) were observed. Partial promoters containing sites IA, IB, and IC (SHΔ−240-92R) or the two CCAAT sites (SH-147R) or sites III and IV (SH−226R) or sites IC, II, and III (SH−273R) were each sufficient for correct spatial regulation. Examples of labeling patterns observed for SHΔ−240-92R and SH−147R are shown in Figs. 2B and 2C and 2D and 2E, respectively. Because the smallest promoter tested, containing sequence from −66 to +20, showed barely detectable β-gal activity (data not shown), its spatial pattern could not be determined.

These results lead to the surprising conclusion that many different combinations of small subregions of the *SpHE* promoter can support nonvegetal transcription. The possibility that each of these subregions also includes a *cis*-element conferring negative regulation in vegetal cells is remote. However, these experiments do not exclude the possibility that the *SpHE* basal promoter and closely adjacent sequences, which were present in all thirteen constructs shown in Figs. 1A and 1B, might be responsible for suppressing *SpHE* transcription in vegetal cells.

The SpHE Basal Promoter Can Function in Vegetal Cells

Recent studies show that basal promoter/initiator sequences can mediate promoter selectivity (reviewed by Novina and Roy, 1996). In the sea urchin, the *SM50* TATA-less promoter contains a *cis* element, site C (+10 to +25), adjacent to the initiator region, which is critical for PMC-specific expression (Makabe *et al.*, 1995). To test whether *SpHE* sequences surrounding +1 could be responsible for nonvegetal transcription of this promoter, we inserted *SpHE*

FIG. 2. Transcription patterns of *SpHE*-pNL constructs in sea urchin midgastrula-stage embryos. (A) The normal domain of *SpHE* expression is in nonvegetal cells of early blastulae, which are progenitors of ectoderm and endoderm tissues, shown labeled in blue. Vegetal cells, which are defined here as PMCs, do not transcribe the *SpHE* gene and are labeled in red. (B–K) Whole mounts of embryos are shown photographed under DIC illumination (B, D, F, H, J) to illustrate patterns of β-gal reporter expression (blue). The same embryos (C, E, G, I, K, respectively) are shown after immunofluorescent detection of PMCs using the 6e10 monoclonal antibody coupled to Cy3 (red). Examples of the labeling patterns observed for SHΔ−240-92R (containing only sites IA–IC) and SH-147R (containing only the CCAATCA sites V and VI) are shown in B and C and D and E, respectively. All partial *SpHE* promoters listed in Figs. 1A and 1B showed similar labeling restricted to nonvegetal cells. (F, G) A typical pattern of expression of the SS−91 construct, demonstrating that the *SpHE* basal promoter is functional in PMCs when driven by PMC-specific elements of the *SM50* promoter. Arrowheads mark the position of doubly labeled PMCs. (H–K) An example of the labeling pattern observed for embryos expressing the SS−1255 *SpHE-SM50* chimeric promoter. H and I and J and K show images of the same embryo at two different focal planes. The PMC ring, stained red, contains several cells expressing the β-gal reporter (stained blue in H) whose positions are indicated in I (arrowheads). A cluster of labeled ectoderm cells is shown in J and its position is indicated in K (arrowhead). The bar in B represents 20 μm.

sequence between −91 and +96 into the PMC-specific *SM50* promoter, replacing the *SM50* sequence between −10 and +29 (Fig. 1C). Expression of this construct (SS−91) was predominantly vegetal and very similar to that of the wild-type *SM50*-PNL promoter: The average number of β-gal-expressing PMCs/embryo was similar, as was the frequency of embryos containing labeled PMCs (about 20%). An example of expression in PMCs by this construct is shown Figs. 2F and 2G. Although the *SM50-SpHE* chimera lacks site C, which has been shown to be absolutely required for *SM50* transcription (Makabe *et al.,* 1995), our data show that the inserted *SpHE* sequence can efficiently replace its function. This result also implies that the *SpHE* basal promoter/initiator region lacks sequences that prevent its activity in vegetal cells and that it can function in concert with vegetal-specific *SM50* regulators.

Unlike wild-type *SM50*-PNL, the SS−91 construct also sponsors a low level of expression in nonvegetal cells (~5%). It seems likely that the inserted *SpHE* sequences, and not the *SM50* elements, mediate expression in these cells because our previous experiments showed that these *SpHE* sequences alone can drive expression of a CAT reporter at low levels (Wei *et al.,* 1995). Furthermore, in an exhaustive analysis, only positive *SM50 cis* elements have been identified in the *SM50* promoter, suggesting that the corresponding transcription factor activities are vegetally restricted (Makabe *et al.,* 1995). Unfortunately, when the −66 to +96 *SpHE* region was tested in assays with the β-gal reporter, activity was too low to determine the spatial pattern of transcription accurately. Therefore, although a small region surrounding the core promoter of *SpHE* can function in vegetal cells with *SM50*-specific factors, it is not known whether it could do so independently.

The SpHE Regulatory Region Does Not Suppress SM50 Promoter Activity

The above data suggest that nonvegetal *SpHE* transcription, like vegetal *SM50* transcription, is regulated by positive rather than negative transcription factor activities. If both *SpHE* and *SM50* are regulated only by positive activities, then these activities should be concentrated in reciprocal territories along the A–V axis and function independently of each other. If this is true, then a promoter construct containing both *SpHE* and *SM50* regulatory elements should be expressed in both vegetal and nonvegetal regions of the embryo. We linked *SpHE* sequence from −1255 to −33 (lacking the *SpHE* basal promoter) immediately upstream of the intact *SM50* regulatory region (SS−1255) and monitored the β-gal expression pattern. An example of an embryo expressing SS−1255 in both vegetal and nonvegetal domains is shown in Figs. 2H–2K. The expected percentage of embryos (~25%) contained labeled PMCs, showing that *SpHE* upstream promoter sequences do not suppress *SM50* promoter activity in this arrangement. Approximately 40% of the embryos had β-gal-positive cells in ectoderm and/or endoderm, which is about half the frequency observed when these same upstream *SpHE* sequences drive the *SpHE* basal promoter. There are two possible major reasons for reduced nonvegetal expression levels. First, the *SpHE* activation elements lie much farther upstream of the *SM50* initiation site in SS−1255 than they do in the *SpHE* gene. Second, *SpHE* regulatory factors might function more efficiently in combination with the *SpHE* basal promoter than with the *SM50* initiation region: The *SpHE* basal promoter contains a TATA box, but the *SM50* basal promoter does not. Although formally our data do not exclude the possibility that *SM50 cis* elements confer some mild negative regulation on *SpHE* promoter activity, extensive mutagenesis of the *SM50* regulatory region has not identified any such element (Makabe *et al.,* 1995). Therefore, we favor the view that, because the activity of SS−1255 is ubiquitous, there is no reciprocal repression between *SpHE* and *SM50* promoter sequences. Instead the simplest interpretation is that positive activities partitioned in the early embryo are responsible for the nonvegetal and vegetal expression patterns of these two genes. Summation of positive activities has also been observed for chimeras between the *SM50* and *Endo16* promoters (Kirchhamer *et al.,* 1996a).

DISCUSSION

The SpHE Regulatory Region Contains Many cis Elements That Mediate Activation of Transcription in Nonvegetal Cells of Early Sea Urchin Embryos

In this work, we provide evidence that the nonvegetal transcription of *SpHE* is controlled predominantly, and probably exclusively, by positive elements and not by negative elements that mediate repression in cells originating at the vegetal pole. Each of the dozen partial *SpHE* promoters, even those with the majority of the regulatory region removed, could drive nonvegetal transcription. Yet all of these, which collectively encompass the entire regulatory region, yielded only very low levels of ectopic expression of a reporter gene encoding β-galactosidase. We believe that these levels of *SpHE* promoter activity in PMCs are attributable primarily to noise level of the assay, as well as to some variability in the boundary between the vegetal and nonvegetal domains of the egg and early embryo. In fact, in a low percentage of embryos, *SpHE* transcription can extend even into the micromere lineage, which may contribute to the low frequency of ectopic expression observed for *SpHE* promoter transgene assays. In contrast, similar assays of the *SM50* PMC-specific promoter and of two chimeric *SM50*/*SpHE* promoters showed expected levels of expression in PMCs, demonstrating that the assay is sufficiently sensitive to detect expression in these cells. These results are consistent with our previous observations that the *SpHE* regulatory sequence contains many positively acting elements, several combinations of which can independently provide high-level transcription (Wei *et al.,* 1995). These elements appear to be redundant with respect to not only promoter

strength but also, as shown here, spatial activity of transgenes.

Several of the factors that bind these partial promoters are general transcription factors, such as ets, CCAAT, and SpOtx (Wei *et al.*, 1995). CCAAT binding proteins are involved in regulating genes transcribing products required in dividing cells of the sea urchin embryo. For example, both of two adjacent CCAAT sites are essential for high-level activity of the cyclin B promoter. The sequence of these *cis* elements, as well as their methylation interference footprints, suggests that they bind the CP1 class of CCAAT factors (Thatcher *et al.*, 1995). It is likely that the CCAAT elements in *SpHE* bind the same factors because site V is an 18/19 match to the consensus CP1 class recognition motif and site VI binds the same factor(s) (Wei *et al.*, 1995). The early histone H3 gene regulatory region also contains a CCAAT motif (DiLiberto *et al.*, 1989) that is likely to bind the same factors. Although all blastomeres of the sea urchin embryo divide approximately synchronously for the first 4 cleavages, it is relevant to note that the 8 progeny of the four 16-cell stage micromeres differ in their cell cycling in an animal–vegetal gradient: Progeny in the upper PMC lineage divide more slowly than the blastomeres above them, to yield 32 PMCs that do not divide again until well after ingression (reviewed by Davidson, 1986). The lower, most vegetal small micromeres divide only once more during cleavage, and withdraw from cycling until several days later at the pluteus larva stage. Thus, numbers and rates of cell division are lowest in the most vegetal region of the embryo. This is consistent with the hypothesis that their cycling could ultimately be limited by the complement of specific activators of transcription of genes such as cyclin and histone. Consistent with this view, small *SpHE* partial promoters, in which the only identified *cis* elements are CCAAT boxes (SH-147 and SH-147R) do not drive transcription in vegetal cells. Although we have searched for additional *cis* elements by EMSAs, no additional specific element has been identified. Clearly, determination of which factors bind the CCAAT elements and where their activities are located will be required to resolve this interesting question.

The finding that the *SpHE* regulatory region contains many positively acting *cis* elements does not exclude the possibility that the corresponding *trans*-acting factors are present in vegetal cells, but in inactive form. For example, SpOtx, which can bind to sequences in the site I region of the *SpHE* promoter, is known to activate *Spec 2A* transcription in ectoderm (Mao *et al.*, 1994) as well as *Endo16* transcription in endoderm cells (E. Davidson, personal communication). In both of these cases, elements binding SpOtx factors appear to be sufficient to mediate specific transcription in the later embryo in either ectoderm or endoderm, two nonvegetally derived tissues in the classification used here. However, SpOtx protein is also found in nuclei of cells of the PMC lineages (Mao *et al.*, 1996) where it presumably is inactive. Thus, Otx function must be controlled by other factors and illustrates that similar kinds of interactions could regulate *SpHE* promoter spatial activity.

SpHE and SM50 Represent the Two Earliest, Cell Autonomous, Spatially Regulated Domains of Transcription along the A–V Axis: Vegetal and Nonvegetal

We propose that an early regulatory event in sea urchin embryos is the establishment of a maternally sponsored nonvegetal domain of gene activity, regulated by the asymmetric disposition of regulatory activities in the egg and/or early embryo, and revealed by the spatial pattern of accumulation of the VEB mRNAs. This domain is physically separated, at least approximately, by the unequal cleavage in the 4 vegetal blastomeres of the 16-cell embryo shortly after transcription of the VEB genes is activated (Reynolds *et al.*, 1992). VEB genes are transcribed in the larger, more animal macromeres, but not in the vegetal micromeres (Reynolds *et al.*, 1992; Nasir *et al.*, 1995). Their transcription in the nonvegetal domain is cell-autonomous (Reynolds *et al.*, 1992; Ghiglione *et al.*, 1993), unlike the subsequent specification of nonvegetal blastomere progeny, which depends heavily on inductive interactions. In contrast, micromeres are distinct in developmental capacity and function: They are the only cells determined at the 16-cell stage and they are the origin of inductive influence in the embryo; in isolation or in any ectopic position, they will assert their maternally determined skeletogenic fate (Hörstadius, 1973). Thus, the activity of vegetal-specific genes, like that of the VEB genes, is governed by cell autonomous processes.

We have previously demonstrated that the border of the nonvegetal region at the time of maximal *SpHE* transcription is not precise, and proposed that it therefore cannot correspond to a border between early blastomere lineages. Together with the fact that activation of the VEB genes is cell-autonomous, this suggests that activation of the VEB genes does not correspond to specification of ultimate cell fates, but instead represents an initial provisional specification of a nonvegetal region. Consistent with this, the *SpHE* gene encodes the hatching enzyme, which lacks a cell-type-specific function. The embryo only requires that the hatching enzyme be synthesized in sufficient quantity within a few hours after VEB stage, which is achieved by transcription at an exceptionally high rate (Reynolds *et al.*, 1992; Wei *et al.*, 1995). The early nonvegetal activation of the *SpHE* gene appears to take advantage of transcriptional activators stockpiled during oogenesis that initially are present, and probably subsequently synthesized, in greater amounts in nonvegetal blastomeres. The *SpHE* regulatory apparatus, with its large number of positive *cis* elements may have evolved to exploit the most abundant maternal positive transcription factors that may also drive early expression of other nonvegetally expressed genes. We have analyzed one of these (*SpAN*) and find that it too contains many positively acting elements, but shares with *SpHE* only two, including

a CCAAT motif that is recognized by the same factor (Kozlowski *et al.*, 1996).

The absence of negative territorial regulation is also observed for the *SM50* gene, when it is activated several cleavages after *SpHE* (Makabe *et al.*, 1995). In contrast, the spatial regulation of genes in different transcriptional territories at later stages appears to be achieved largely by negatively acting factors (for example, Hough-Evans *et al.*, 1990; Nemer *et al.*, 1995; Frudakis and Wilt, 1995; Xu *et al.*, 1996; reviewed by Kirchhamer *et al.*, 1996b). These observations lead to the model that very early developmental differences in gene activity are set up by segregation of maternal positive transcriptional activities along the animal–vegetal axis whereas refinements in pattern in subsequently established territories rely on the emergence of negative regulatory activities in the embryo.

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